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PRINCIPAL INVESTIGATOR: Lance R. Thomas

CONTRACTING ORGANIZATION: Wake Forest University School of Medicine
Winston-Salem, North Carolina 27157

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6. AUTHOR(S)
Lance R. Thomas

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Wake Forest University School of Medicine
Winston-Salem, North Carolina 27157

E-Mail: lanthoma@wfubmc.edu

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13. ABSTRACT (Maximum 200 Words)

Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block apoptosis induced by death ligands in many cell lines. While studying FADD signaling, our laboratory made the surprising discovery that FADD-DN can induce apoptosis in normal breast epithelial cells. Because FADD-DN induces apoptosis in normal but not cancerous breast epithelial cells, we hypothesize that FADD-DN interacts with one or more proteins expressed in breast epithelia. Since breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis. Since defects in apoptotic pathways are a prerequisite to cancer, understanding the nature of these defects may bring about potential treatments. FADD-DN signaling presents a novel apoptotic pathway that is fundamental in normal breast epithelia, but not breast cancer cells. Components of this pathway may identify potential therapeutic targets that allow the reactivation of this apoptotic response in cancer cells

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Introduction

Normal cells undergo apoptosis in response to inappropriate growth signals or the lack of overt survival signals. Tumor cells possess defects in apoptosis regulatory pathways and do not undergo apoptosis in these situations. There are two modes of apoptosis - an intrinsic pathway initiated by stress such as DNA damage and an extrinsic pathway resulting from activation of death receptors. Binding of ligand to a death receptor such as Fas, TNFR1 or TRAIL receptors 1 and 2 leads to activation of that receptor. This results in the recruitment of the cytoplasmic adaptor protein FADD to the receptor complex and activation of caspase-8. Because FADD is an essential component of receptor mediated apoptosis, a dominant-negative version (FADD-DN) is able to block both Fas and TNF induced apoptosis in many cell lines. However, experiments in our lab indicate that FADD-DN can kill normal human breast epithelial cells but not breast tumor cells. Since the only known role of FADD is an adaptor molecule, our hypothesis is that FADD-DN induces apoptosis in normal breast cells through interactions with one or more proteins expressed in breast epithelia. Our approach is to identify proteins that bind to FADD then identify the subset that are involved in FADD-DN binding using mutational analysis. Because breast tumor cells do not die in response to FADD-DN, the potential FADD-DN interacting partners are likely to be involved in carcinogenesis.

Body

We have achieved all the objectives described in the approved statement of work for year two. Previously we identified 17 FADD-DN interacting proteins using a yest two-hybrid approach. We subsequently narrowed this to two candidates by testing for interaction in mammalian cells by immunoprecipitation. The first is the death receptor DR5 which binds to the cytotoxic ligand TRAIL. This identification of DR5 was interesting because previous reports indicate that TRAIL is able to kill normal cells but not tumor cells (*1*). In addition, a recent report from Mills et al. suggests that TRAIL induced apoptosis is an important developmental step in the formation of mammary lumen formation (2). Defects in this pathway may contribute to the early events of breast cancer development. The second candidate is a protein with no known function, PL31. The scope for the second year of work was to determine which of these binding partners is involved in FADD-DN induced death. To accomplish this task we proposed to use our yeast reverse two-hybrid system (*3*) to identify mutations in FADD-DN that prevented interaction with a given binding partner. We can then test the ability of the mutant FADD-DN to kill normal cells. A mutant FADD-DN that cannot bind to a given protein and does not kill normal cells likely indicates that this protein is involved in FADD-DN induced death.

To test the efficacy of our approach, we screened for mutations in FADD-DN that prevent binding to know FADD binding partner, Fas but still allow interaction with DR5. A mutation of valine 108 to glutamate prevents binding of FADD-DN to Fas but does not destroy overall protein structure because this molecule is still able to bind DR5 (figure 1a). To test whether the the mutations that prevented binding in yeast could also prevent binding in mammalian cells, we assessed the the response of FADD-deficient cells stably expressing wild type FADD of FADD (V108E) to Fas ligand (FasL) or TRAIL. One of the first events in death receptor signaling is the cleavage of caspase-8 into its active

subunits which is indicitave of caspase activation. Figure 1b shows that FADD (V108E) is able to transduce TRAIL signaling but not FasL signaling. Thus mutations in FADD that prevent binding in yeast also prevent binding in mammalian cells.

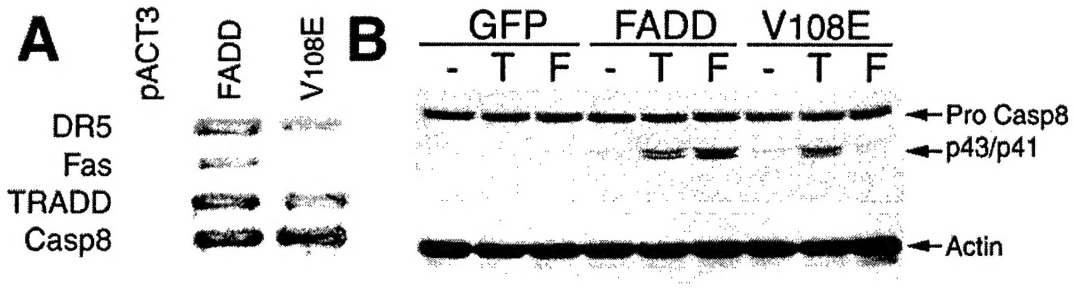

Figure 1: A mutation of valine 108 to glutamate prevents binding to Fas but not other FADD binding partners. (A) Wild type FADD is able to interact with DR5, Fas, TRADD, and caspase-8 in yeast while the V108E mutation prevents binding to Fas only. pACT3 is empty vector and a change to blue indicates interaction. (B) Caspase processing was measured in FADD deficient cells expressing wild type FADD or FADD (V108E) in response to TRAIL (T) or Fas Ligand (F). FADD (V108E) is not able to transduce Fas signaling indicating that it is not able to bind Fas in mammalian cells.

Using a directed yeast two-hybrid assay, we screened through current FADD-DN mutations and used our yeast reverse two-hybrid system to identify additional mutations. Mutations in FADD-DN that prevent binding to DR5 were R113E, R117A, D123R, R135E and D175R; D106A, D140A and R166E showed decreased binding (figure 2). D106A, R117P, V121N, and D175A reduce binding of FADD-DN to PL31.

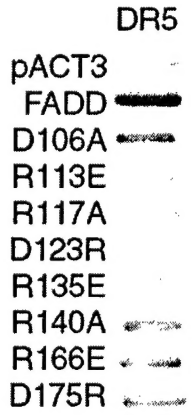

Figure 2: Mutations in FADD-DN that prevent binding to DR5. DR5 was tested for interaction with various FADD mutants using a directed yeast two-hybrid approach. Many of the mutations prevent binding to DR5 (R113E, R117A, D123R, R135E, D175R) while some decrease binding (D106A, R140A, and R166E). pACT3 is empty vector and a change to blue indicates interaction with DR5.

We tested several of the FADD-DN to see whether the mutations prevented FADD-DN induced death. FADD-DN with mutations at arginine 117 or aspartate 175 did not kill normal cells suggesting a role for DR5. The situation with PL31 is more complex because most of the mutations in FADD-DN decrease, but do not prevent binding to PL31.

We reasoned that both DR5 and PL31 might be involved in FADD-DN induced death because both are able to bind FADD *in-vivo*. In addition we have previously shown that PL31 kills normal cells but not tumor cells similar to FADD-DN. We therefore tested the ability of FADD to mediate an interaction between DR5 and PL31 in a directed yeast three-hybrid assay. As a positive control, we tested for a known function of FADD which is to mediate the interaction between Fas and caspase-8. FADD was able to mediate an interaction between DR5 and PL31 (figure 3). This suggests that both DR5 and PL31 might be involved in FADD-DN induced death.

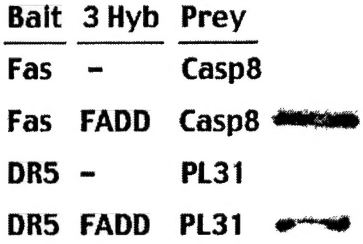

Figure 3: FADD is able to mediate an interaction between DR5 and PL31. We used a yeast three-hybrid approach to test whether FADD can mediate an interaction between DR5 and PL31. An empty three-hybrid vector results in no color change while yeast with a three hybrid vector expressing FADD turn blule indicating that FADD can mediate binding of DR5 to PL31. As a positive control, FADD was tested for its ability to mediate an interaction between Fas and caspase-8.

Key Research Accomplishments

We demonstrated that mutations in FADD that pevent interaction with a given binding partner identified using our yeast reverse two-hybrid system also prevented binding in mammalian cells.

We generated plasmids to use in a reverse two-hybrid screen.

We identified mutations in FADD-DN that prevent binding to our two candidate proteins, DR5 and PL31.

We constructed plasmids expressing these FADD-DN mutations and tested the abiliity of several mutations to kill normal cells.

Manuscripts:

Thomas, L. R., Henson, A., Reed, J. C., and Thorburn, A. Direct binding of FADD to the TRAIL receptor DR5 is regulated by the death effector domain of FADD. In revision, *The Journal of Biological Chemistry.*

Thomas, L. R., Johnson, R. L., Reed, J. C., Freddie R. Salsbury and Thorburn, A. The C-terminal Tail of TRAIL receptors DR4 and DR5 regulates the response to apoptotic stimuli. In revision, *Molecular Cell.*

Abstracts:

May 2003: The Susan G. Komen Breast cancer foundation. *A cell death pathway that is defective early in cancer development.*

June 2003: The 19th Annual Meeting on Oncogenes. *Direct binding of FADD to TRAIL receptors DR4 and DR5 requires regions outside the core death domain.*

September 2003: Cold Spring Harbor meeting on Programmed Cell Death. *Direct binding of FADD to TRAIL receptors DR4 and DR5 requires regions outside the core death domain.*

Conclusions

To test the efficacy of our yeast reverse two-hybrid system, we identified mutations in FADD that prevent binding to Fas but not DR5 and tested these mutations for the ability to transduce signaling through DR5 but not Fas. We identified a series of mutations in FADD-DN that prevent binding to DR5 and PL31, two candidate proteins we believe to be important in FADD-DN induced death. Several of the mutations that prevent binding to DR5 do not kill normal cells indicating that DR5 might be involved in FADD-DN induced death. In addition, it appears that FADD can mediate an interaction between DR5 and PL31 suggesting a role for both proteins in FADD-DN induced death.

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3. L. R. Thomas, D. J. Stillman, A. Thorburn, *J Biol Chem* 277, 34343-8 (Sep 13, 2002).